Vibrio species are predominantly intracellular within cultures of Neoparamoeba perurans, causative agent of Amoebic Gill Disease (AGD)

Colour print requested for figure 2 and figure 3

Vibrio species are predominantly intracellular within cultures of *Neoparamoeba perurans*, causative agent of Amoebic Gill Disease (AGD)

David P.C. MacPhail[1], Rhea Koppenstein[1], Sutherland K. Maciver[2], Richard Paley[3], Matt Longshaw[4], Fiona L. Henriquez[1*]

[1]Institute of Biomedical and Environmental Research, School of Health and Life Sciences, University of West of Scotland, High Street, Paisley, PA1 2BE

[2]Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh Medical School, Biomedical Sciences, Hugh Robson Building, 15 George Square, Edinburgh, EH8 9XD

[3]Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB

[4]Benchmark Animal Health Ltd., Bush House, Edinburgh Technopole, Milton Bridge, Edinburgh, EH26 0BB

[*]corresponding author: Fiona.henriquez@uws.ac.uk

Keywords: *Paramoeba*, amoebic gill disease, bacteria, *Vibrio*, 16S

Acknowledgements: DPCM was funded through a PhD studentship 50% University of the West of Scotland and 50% Benchmark Animal Health

Abstract

Neoparamoeba perurans is a free-living protist that can cause Amoebic Gill Disease (AGD) in a number of teleost fish species and is responsible for substantial losses of farmed Atlantic salmon in various locations world-wide. The intimate relationship of the amoeba with bacteria can present challenges for its laboratory culture and drug discovery programmes. Herein, we report our findings on the bacteria that live in close association with *N. perurans*. These include the presence of various marine bacteria, including those of the *Pseudoalteromonas*, *Halomonas*, *Cellulophaga* and *Mesonia* genera. However, next generation sequencing (NGS) identified a substantial proportion of sequences that matched with the *Vibrio* genus in filtered amoebae and not in the medium suggesting an intimate association between this genus and *N. perurans*. Fluorescence *in-situ* hybridization (FISH) revealed that *Vibrio* species are predominantly found within *N. perurans*. This information is important in the management and control of AGD as bacteria associated with *N. perurans* may have relevance to virulence and advancement of disease.

1. Introduction

Neoparamoeba perurans is a free-living protist that can cause Amoebic Gill Disease (AGD) in a wide range of teleost fishes, and is responsible for substantial losses of farmed Atlantic salmon (*Salmo salar*), with incidence of *Neoparamoeba* infection detected in Australia, New Zealand, Japan, Chile, USA, Scotland, Ireland, France, Spain and Norway (Bustos et al., 2011; Crosbie et al., 2010; Oldham et al., 2016; Steinum et al., 2008; Young et al., 2008b). The impact of AGD may also be accentuated by the consequences of climate change, such as sea temperature, supply of nutrient, harmful algal blooms (Foyle et al., 2020). *N. perurans* colonizes the fish gill epithelium, and causes structural changes in this tissue, which can increase morbidity and mortality of its host (Cano et al., 2019). This is problematic for the aquaculture industry, as it requires costly ongoing management and treatment of the disease, as the infection recurs due to the limited immune response (Young et al., 2008a). Despite these difficulties, currently relatively little is known about this opportunistic pathogen, and the disease it causes.

Neoparamoeba perurans and AGD research is complicated by the inability to develop axenic cultures (Figure 1) (Collins et al., 2017). The sustained presence of bacteria in *Neoparamoeba* cultures is problematic, as the presence of bacteria can affect the ability to perform high-throughput screening of drug compounds against *Neoparamoeba* and to understand its metabolic pathways. In addition, the presence of bacteria may also hinder the outcomes of colorimetric or other biochemical cell-based assays, necessitating manual cell counting methods to assess potential inhibitory compounds, which is time consuming. Furthermore, bacteria within the culture may be pathogenic to the amoeba, leading to partial or total collapse of the culture; any treatment used to reduce bacterial loads could further interfere with any assays conducted.

While the bacterial contamination may be problematic from a research point of view, it is important to consider the basic predator phagocytic relationship and also the potential symbiotic relationship between the amoebae and the bacteria, as bacteria are known to produce compounds which can be utilised by eukaryotes. Examples of the latter include the interactions between bacteria such as *Flavobacterium*, *Roseobacter*, and *Sulfitobacter* spp. and diatoms (Amin et al., 2012) or the suspected symbiotic relationship between algae and bacteria, where algae, including seaweed species acquire Vitamin B_{12} from bacteria to produce methionine (Croft et al., 2005). On the other hand, bacteria may also benefit from these relationships, such as protection from environmental risks and (Thomas et al., 2010) as is the case with *Candidatus Legionella jeonii* and *A. proteus* (Park et al., 2006). It is therefore possible that some species of bacteria may have a symbiotic relationship with *Neoparamoeba* spp., providing compounds required for amoeba growth, or acting as a food source for the amoeba. There has previously been focus on the bacterial microbiome associated with *N. perurans*, such as the recent study of the effects of temperature on *N. perurans* and the microbial community within *in vitro* cultures of the pathogen (Benedicenti et al., 2019). While this study did not elucidate the relationship between the bacteria and *N. perurans,* it is important this relationship is further refined in order to develop potential new control measures, to improve culture techniques and to further our understanding of symbiosis.

Bacteria can also be fish pathogens and their association with other pathogens, such as *N. perurans* may be relevant to emergence of complex gill disease (CGD), a multi-pathogen

disease used to describe a nonspecific gill condition. The aim of this study is to describe the variety of different bacteria associated with *N. perurans,* which may impact on virulence of *N. perurans* and its role in CGD (Herrero et al., 2018).

2. Methods

2.1 Neoparamoeba perurans culture

Neoparamoeba perurans was utilised from a long-term culture originally isolated from farmed Atlantic salmon from the west coast of Scotland. It was cultured under sterile conditions under a class II safety cabinet to avoid any environmental contamination in T75 vented-cap tissue culture flasks (VWR, Leicestershire, England) in Malt-Yeast Broth (MYB) [0.1g/l Malt Extract (Oxoid™, ThermoFisher Scientific, Renfrew, Scotland), 0.1g/l Yeast Extract (Oxoid™, ThermoFisher Scientific), and filtered 35 PSU Peacock Salt Seamix Artificial Sea Water (J C Peacock & Co Ltd)]. The amoebae were incubated at 18°C, and the medium was changed weekly, in order to prevent overgrowth of bacteria present in the culture. In order to maintain healthy amoebae, the cells were subcultured by mechanically detaching them from the surface of the flask and transferring to a new T75 flask containing medium as described above. The MYB culture medium was changed the following day, once cells had adhered to the surface.

2.2 Isolation and Identification of bacterial species

Bacterial species were isolated from the *N. perurans* cultures by suspending the amoeba cultures in MYB and performing 1 in 10 serial dilutions. The serial dilutions were cultured on Luria Bertani (LB) Agar + 75% seawater plates [35g/l LB agar powder (Oxoid™, ThermoFisher Scientific), 750ml/L 35 PSU artificial seawater, 250ml/L distilled H_2O] and incubated at 25°C and 37°C. Individual colonies were selected for Gram staining and culture in LB broth + 75% seawater [20g/l LB broth powder (Oxoid™, ThermoFisher Scientific), 750ml/L 35 PSU artificial seawater, 250ml/L distilled H_2O]. The isolated cultures were used to perform genomic DNA (gDNA) extraction, to allow identification of the individual bacterial isolates. Gram staining was performed to observe the cells under a microscope and identify their morphology. Stocks of the bacterial isolates were preserved through cryopreservation (500µl of culture was added to 500µl of 50% glycerol w/LB broth + 75% seawater) and stored at -80°C.

2.3 Genomic Nucleic Acid Extraction

DNA extraction was performed on the isolated bacteria and *N. perurans* filtered culture. *N. perurans* was collected by centrifugation for 8 minutes at 3000 × *g* to pellet the cells and remove the MYB culture media. The cells were suspended in 35 PSU filtered artificial seawater, then the centrifugation step and wash were repeated an additional two times, discarding the supernatant each wash. The cells were resuspended in 5ml of 35 PSU filtered artificial seawater and passed through a 0.45µm pore size filter, selected as there is evidence that a significant proportion of the seawater bacterial microbiome can pass through a 0.45µm pore size (Denner et al., 2002), allowing the *N. perurans*, which is 10-20µm in diameter, to be retained.

For the bacteria, single colonies were grown in LB broth with 75 % artificial seawater at 25°C, with shaking at 225rpm for 24 to 48 hours. The bacteria were harvested at 3000 × *g* for 10 minutes, and the supernatant was discarded. DNA extraction was performed using the tri-reagent® (Life Technologies, Renfrew) phenol-chloroform separation method according to the tri-reagent® protocol. The method was modified to omit the addition of EDTA, which can interfere with the PCR reaction (Huggett et al., 2008). The gDNA was then quantified on a NanoDrop 1000 spectrophotometer (Thermofisher Scientific), and stored at 4°C for future use.

2.4 Polymerase Chain Reaction (PCR)

PCR reactions consisted of 12.5µl DreamTaq (2x PCR mastermix) (Thermofisher Scientific,), 50 pmol forward and reverse 16S universal oligonucleotide primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG -3'), S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC -3') (Klindworth et al., 2013) (Standard Oligos, Thermofisher Scientific,), 11µl nuclease-free water (Thermofisher Scientific), and 1µl of template. The PCRs were performed with an initial denaturation at 94°C, followed by 40 cycles of denaturation at 94°C, annealing at 55°C for 16S universal oligonucleotide primers (template extracted bacterial genomic DNA) and extension at 72°C, with a final extension at 72°C. The PCR products were visualised on a 2% agarose gel, run for 45 minutes at 125v, 400mA and viewed with a transilluminator following ethidium bromide (EtBr) (Sigma-Aldrich Company Ltd, Irvine, Scotland) staining.

2.5 Sequencing

PCR-amplified DNA fragments were isolated from EtBr stained agarose gels via PureLink™ Quick Gel Extraction Kit (Thermofisher Scientific,). The purified PCR amplified products were ligated into the pCR®4-TOPO® vector, using the TOPO™ TA Cloning™ Kit for Sequencing (Thermofisher Scientific) according to the manufacturer's instructions. Competent DH5α were transformed with 5µl of the ligation reaction using the heat shock method, as described in the manufacturers protocol. Transformed cells were then spread evenly onto LB agar (Oxoid™, ThermoFisher Scientific), which had been previously coated with 100 µg/ml ampicillin, and incubated overnight at 37°C. Successful transformants underwent plasmid purification using the PureLink™ Quick Plasmid Miniprep Kit (Thermofisher Scientific,), according to the manufacturer's instructions. Sanger sequencing of PCR amplified products was achieved using the M13 uni (-21) or T7 primers, and was carried out commercially by Eurofins Genomics, Ebersberg, Germany. Nucleotide BLASTn was performed to identify the bacteria isolated from the *N. perurans* culture.

2.6 Next Generation Sequencing (NGS)

Cultures of *N. perurans* were prepared to allow the collection of DNA to be performed at various stages and culture conditions. These conditions were Day 0 (day of subculture), Day 1 of culture, Day 1 with 1% Penicillin-Streptomycin (PS) (Thermofisher Scientific), Day 7, 1

month of culture, and filtered amoebae from the day 1 culture (Bottle top vacuum filtration systems, PES, VWR). All samples were collected in TRI Reagent® (Thermofisher Scientific,). DNA extraction was performed using the TRI Reagent® phenol-chloroform separation method, suspended in nuclease-free water, and the extracted DNA was quantified using the NanoDrop 1000 spectrophotometer. 50µl of DNA samples at a concentration of 50ng/µl were then loaded into designated wells of a 96-well plate and transferred to Eurofins Genomics (Ebersberg, Germany) for illumina sequencing of the V1-V3 region of the 16S ribosomal RNA gene (Allen et al., 2016) and bioinformatic analysis.

2.7 Fluorescence In Situ Hybridisation (FISH)

A total of $3x10^5$ *N. perurans* were collected by centrifugation as described in paragraph 2.3. The cells were resuspended in 1ml of 35 PSU filtered artificial seawater and pipetted into a 24-well plate (333µl per well) (Ibidi µ-Plate 24-well plates treated with ibiTreat, Thistle Scientific, Glasgow, Scotland). The cells were left to adhere to the surface of the plate at room temperature for 1 hour. Once attached, the cells were fixed using a 4% formaldehyde with artificial seawater mix and incubated at room temperature for 15 minutes. Following the incubation, the cells were washed with artificial seawater. The artificial seawater was discarded, followed by 3-minute room temperature incubations using 50%, 80%, and 96% ethanol (EtOH) with artificial seawater. The 96% ethanol supernatant was removed, and the cells were washed with hybridisation buffer [20mM Tris (pH 9.0), 100mM NaCl, 0.5% SDS, 0.2µm filtered]. The hybridisation buffer wash was removed and replaced with hybridisation buffer containing 200nM cyanine-5 labelled *Vibrio* 16S-1 PNA probe (5'-AGGAGCTTCGCTTGC-3')(Zhang et al., 2015) (Biomers, Germany). The negative control comprised hybridisation buffer without the PNA probe. The plate was incubated in the dark at 55°C for 1 hour. The hybridisation solutions were removed and replaced with pre-heated (55°C) wash solution [10mM Tris, 1mM EDTA, 0.2µm filtered]. The plate was incubated in the dark, at 55°C for 10 minutes. The wash solution was discarded, and the plate was dried at 70°C. 300µl of 1x Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.6) was added to each well, with gentle agitation to cover the bottom surface of the well. A drop of NeoMount Fluo with DAPI and PG (Fluorescent mounting medium) (Neobiotech, Generon, Slough, England) was added to each well with gentle agitation, then incubated in the dark at room temperature for 10 minutes. Following the incubation, the suspension was removed from each well, and washed

with TBS. Images of the amoeba were recorded using an inverted fluorescent microscope (Olympus IX71 Inverted Microscope, Olympus TH4-200 Light Power Supply, Olympus U-HGLGPS Fluorescence Light Source, Prior Proscan III Motorised Stage Controller) with DAPI and cyanine 5 filters to detect the staining.

3. Results

3.1 Culture and 16S sequencing identify three classes of bacteria associated with N. perurans

Sequencing was successfully achieved for 16 out of the 17 bacterial colonies sampled from LB seawater agar with no filtration, resulting in the identification of at least 9 different Gram-negative species (Table 1 and Table A in Supplementary data) of three classes α-proteobacteria, γ- proteobacteria and *Flavobacteria*. Most colonies identified as belonging to a specific genus, but 16S sequencing did not allow sub-genus classification.

3.2 Next Generation Sequencing (NGS) reveals that Vibrio species are the most abundant genus associated with fresh N. perurans cultures.

With the successful isolation and identification of bacteria associated with *N. perurans* growth on LB agar, it was important to identify bacteria non culturable by this method by using NGS techniques. Proteobacteria was the predominant phylum of the sequences identified in all the culture conditions. A small proportion of sequences were associated with the phylum Bacteroidetes, with the highest proportion identified at day 7 and 1 month of amoeba culture. The filtered amoeba collection predominantly consisted of Proteobacteria (Figure 2).

Analysis of microbes at the genus level of nucleotide sequences indicated that the *Vibrio* genus was found in high proportion for all non-filtered samples at early time points, including in those treated with penicillin and streptomycin, and in the filtered amoebae (Figure 2; Table 2). Various genera within the *Flavobacteriaceae* family, including the genera *Cellulophaga*, *Flaviramulus*, *Mesonia*, and *Muricauda* and a variety of *Rhodobacteriaceae,* including the genera *Labrenzia* and *Pacificibacter* were also identified.

3.3 Vibrio species is predominantly located inside N. perurans

The presence of *Vibrio* inside *N. perurans* was further explored through *FISH* with a *Vibrio* specific probe. *FISH* confirmed that *Vibrio* is predominantly found inside the amoebae and very few were identified in the extracellular medium. This is in contrast to other bacteria that can be identified through DAPI staining (Figure 3).

4. Discussion

Neoparamoeba perurans is a free-living marine amoeba that can cause Amoebic Gill Disease (AGD), which is responsible for substantial economic loss to the aquaculture industry. To date *N. perurans* remains an elusive microorganism in terms of its biology and biochemistry. Drug treatments for AGD rely on *N. perurans* characterisation and the ability to perform high throughput assays *in vitro* before validating any drug treatment *in vivo*. Research into control methods for other diseases caused by amoebae, such as *Acanthamoeba* keratitis, and primary amoebic encephalitis, caused by *Naegleria* species, have benefited from the development of axenic cultures that are free from obvious bacterial growth (Visvesvara et al., 2007). In drug

development axenic culture allows for a detailed analysis of the effect of the drug on the amoeba itself avoiding the effect of confounding factors associated with allied flora and fauna. The development of an axenic culture for *N. perurans* is challenging and despite several attempts to separate the *N. perurans* from its bacteria, it is likely that live, viable bacteria are important to *N. perurans* viability *in vitro*. Since efforts to separate bacteria from the *N. perurans* have not succeeded, it is likely that *P. perurans* is highly dependent on this relationship. Therefore, in this study we aimed to characterize the bacteria intimately associated with *N. perurans* to further the understanding of its lifestyle.

The presence of the bacteria identified in the *N. perurans* cultures may not be casual as studies have reported possible beneficial relationships between these species and eukaryotes. For example, *Pseudoalteromonas* and *Alteromonas* spp. may be associated with the surface of eukaryotes, and are known to produce extracellular inhibitory compounds that inhibit other bacterial growth, such as the AlpP antimicrobial protein. This relationship has been considered as a potential symbiotic mechanism for defence against biofouling in multicellular eukaryotes (such as macroalgae) (Rao et al., 2005). *Halomonas* spp. is known to produce Vitamin B_{12}, which has been demonstrated to be enhanced by adding the algal extract fucoidan (Amin et al., 2012; Croft et al., 2005), suggesting that it be an exogenous source of the vitamin for eukaryotes such as algae.

NGS was performed to determine the presence of any unculturable bacteria. One sample was extracted from a culture supplemented with penicillin-streptomycin. This antibiotic cocktail is widely used to reduce the possibility of bacterial contamination in cell culture. It was used in *N. perurans* culture during the axenization attempts. However, it was observed that supplementation had a detrimental effect on amoeba growth. We originally hypothesised that this may be due to the antibiotic reducing viable bacteria but NGS revealed that this is not likely to be the case. NGS confirmed the presence of the bacteria already identified using classical 16s amplification method, but interestingly also identified the presence of non-culturable *Vibrio* spp. in all culture conditions, and this genus was responsible for a substantial proportion of the microbiome. In our study the identification was limited to genus

identification with some suggested species annotated. FISH revealed that *Vibrio* species are predominantly found inside the amoeba.

Previous work suggests a potential for *Vibrio* to be an endosymbiont. *V. harveyi* has been identified as an endosymbiont of *Cryptocaryon irritans*¸ which causes the parasitic disease of marine cryptocaryonosis and *Vibrio cholerae* is reported to survive within the free-living and opportunistic pathogenic amoeba *Acanthamoeba* spp. and may be released from the amoeba vacuoles by exocytosis (Van der Henst et al., 2016).

Vibrio spp. including *Vibrio tasmaniensis* and *Vibrio splendidus*, have been isolated from Atlantic salmon (Thompson et al., 2003). These are two particular species are likely to be within the microbiome of the *N. perurans* culture (Table B in Supplementary Data)*,* and their isolation from the Atlantic salmon suggest *Neoparamoeba* and *Vibrio* species may interact within the Salmon host. It is interesting to speculate that the gill tissues may be a source of *Vibrio* spp. to the amoeba and that this in turn may contribute to the host providing a suitable environment for *N. perurans*.

Given the strong association with the *in vitro* culture of *N. perurans*, and the *Vibrio* spp. detected by microbiome sequencing, it is possible there is a symbiotic relationship between the bacteria and the *N. perurans* that may influence the development or pathogenicity of AGD*,* particularly as viable bacteria are noted within the amoeba cytoplasm. The symbiotic relationship does not necessarily have to be beneficial to individual amoeba, as amoebae may act as vectors for maintaining the high proportion of *Vibrio* spp. within the bacterial microbiome leading to increased bacterial loads on the fish host, or the *Vibrio* may be beneficial to the overall maintenance and survival of the *N. perurans* population by providing metabolites or cellular functions to its amoeba host. Further investigation into the relationship between *Vibrio* spp. and *N. perurans* is required to determine their relationship and determine whether this could contribute to the pathogenesis of AGD. If a positive relationship is determined, methods could be developed to target the bacteria within the amoeba, thus reducing the overall pathogenicity of the amoeba to its fish host.

We speculate that bacteria present within the same environment, on the surface, or intracellularly within the amoeba may be a symbiont of *Neoparamoeba* spp. and contribute directly, or indirectly to AGD pathogenesis. This potential interaction and contribution is supported by the emergence of complex gill disease (CGD), a multi-pathogen disease associated with *N. perurans* infection, and various other pathogens, including a paramyxovirus, pox virus, phytoplankton, and *Candidatus Branchiomonas cisticola* (Boerlage et al., 2020). AGD and CGD occurrences have coincided, which demonstrates there may multifactorial contributors to AGD and gill health (Herrero et al., 2018). Future work in AGD could focus on the impact of *Neoparamoeba*-bacteria interactions in the emergence of gill disease.

References

Allen, H.K., Bayles, D.O., Looft, T., Trachsel, J., Bass, B.E., Alt, D.P., Bearson, S.M.D., Nicholson, T., Casey, T.A., 2016. Pipeline for amplifying and analyzing amplicons of the V1–V3 region of the 16S rRNA gene. BMC Res. Notes 9, 380. https://doi.org/10.1186/s13104-016-2172-6

Amin, S.A., Parker, M.S., Armbrust, E.V., 2012. Interactions between Diatoms and Bacteria. Microbiol. Mol. Biol. Rev. 76, 667 LP – 684. https://doi.org/10.1128/MMBR.00007-12

Benedicenti, O., Secombes, C.J., Collins, C., 2019. Effects of temperature on Paramoeba perurans growth in culture and the associated microbial community. Parasitology 146, 533–542. https://doi.org/DOI: 10.1017/S0031182018001798

Boerlage, A.S., Ashby, A., Herrero, A., Reeves, A., Gunn, G.J., Rodger, H.D., 2020. Epidemiology of marine gill diseases in Atlantic salmon (Salmo salar) aquaculture: a review. Rev. Aquac. n/a. https://doi.org/10.1111/raq.12426

Bustos, P.A., Young, N.D., Rozas, M.A., Bohle, H.M., Ildefonso, R.S., Morrison, R.N., Nowak, B.F., 2011. Amoebic gill disease (AGD) in Atlantic salmon (Salmo salar) farmed in Chile. Aquaculture 310, 281–288. https://doi.org/10.1016/j.aquaculture.2010.11.001

Cano, I., Taylor, N.G.H., Bayley, A., Gunning, S., McCullough, R., Bateman, K., Nowak, B.F., Paley, R.K., 2019. In vitro gill cell monolayer successfully reproduces in vivo Atlantic salmon host responses to Neoparamoeba perurans infection. Fish Shellfish Immunol. 86, 287–300. https://doi.org/https://doi.org/10.1016/j.fsi.2018.11.029

347 Collins, C., Hall, M., Bruno, D., Sokolowska, J., Duncan, L., Yuecel, R., McCarthy, U., Fordyce,
348 M.J., Pert, C.C., McIntosh, R., MacKay, Z., 2017. Generation of Paramoeba perurans
349 clonal cultures using flow cytometry and confirmation of virulence. J. Fish Dis. 40, 351–
350 365. https://doi.org/10.1111/jfd.12517
351 Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire
352 vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.
353 https://doi.org/10.1038/nature04056
354 Crosbie, P.B.B., Ogawa, K., Nakano, D., Nowak, B.F., 2010. Amoebic gill disease in hatchery-
355 reared ayu, Plecoglossus altivelis (Temminck & Schlegel), in Japan is caused by
356 Neoparamoeba perurans. J. Fish Dis. 33, 455–458. https://doi.org/10.1111/j.1365-
357 2761.2009.01137.x
358 Denner, E.B.M., Vybiral, D., Fischer, U.R., Velimirov, B., Busse, H.-J., 2002. Vibrio calviensis
359 sp. nov., a halophilic, facultatively oligotrophic 0.2 microm-filterable marine
360 bacterium. Int. J. Syst. Evol. Microbiol. 52, 549–553.
361 https://doi.org/10.1099/00207713-52-2-549
362 Foyle, K.L., Hess, S., Powell, M.D., Herbert, N.A., 2020. What Is Gill Health and What Is Its
363 Role in Marine Finfish Aquaculture in the Face of a Changing Climate? Front. Mar. Sci.
364 7, 400. https://doi.org/10.3389/fmars.2020.00400
365 Herrero, A., Thompson, K.D., Ashby, A., Rodger, H.D., Dagleish, M.P., 2018. Complex Gill
366 Disease: an Emerging Syndrome in Farmed Atlantic Salmon (Salmo salar L.). J. Comp.
367 Pathol. 163, 23–28. https://doi.org/10.1016/j.jcpa.2018.07.004
368 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013.
369 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
370 generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1–e1.
371 https://doi.org/10.1093/nar/gks808
372 Oldham, T., Nowak, B.F., Rodger, H., 2016. Incidence and distribution of amoebic gill disease
373 (AGD) — An epidemiological review. Aquaculture 457, 35—42.
374 https://doi.org/10.1016/j.aquaculture.2016.02.013
375 Park, M., Yun, S.T., Hwang, S.-Y., Chun, C.-I., Ahn, T.I., 2006. The dps gene of symbiotic
376 “Candidatus Legionella jeonii” in Amoeba proteus responds to hydrogen peroxide and
377 phagocytosis. J. Bacteriol. 188, 7572–7580. https://doi.org/10.1128/JB.00576-06
378 Rao, D., Webb, J.S., Kjelleberg, S., 2005. Competitive Interactions in Mixed-Species Biofilms

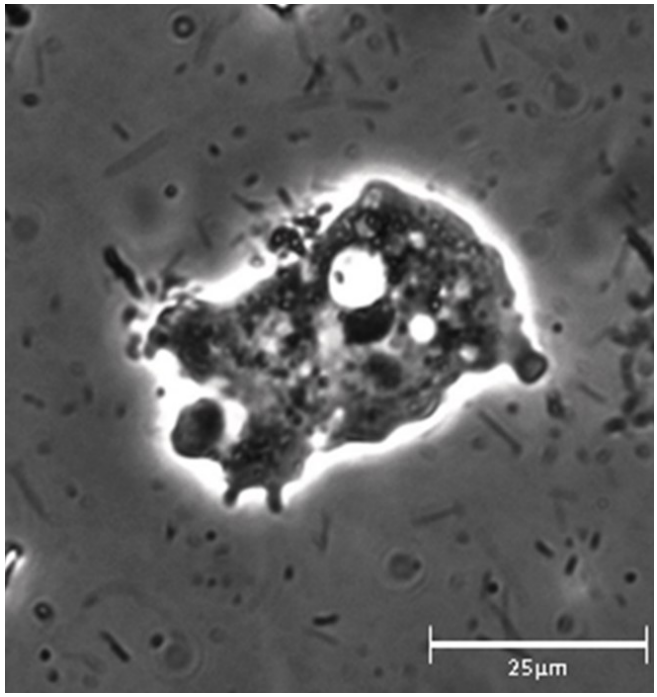
379 Containing the Marine Bacterium Pseudoalteromonas tunicata
380 Appl. Environ. Microbiol. 71, 1729 LP – 1736. https://doi.org/10.1128/AEM.71.4.1729-
381 1736.2005
382 Steinum, T., Kvellestad, A., Rønneberg, L.B., Nilsen, H., Asheim, A., Fjell, K., Nygård, S.M.R.,
383 Olsen, A.B., Dale, O.B., 2008. First cases of amoebic gill disease (AGD) in Norwegian
384 seawater farmed Atlantic salmon, Salmo salar L., and phylogeny of the causative
385 amoeba using 18S cDNA sequences. J. Fish Dis. 31, 205–214.
386 https://doi.org/10.1111/j.1365-2761.2007.00893.x
387 Thomas, V., McDonnell, G., Denyer, S.P., Maillard, J.-Y., 2010. Free-living amoebae and their
388 intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol. Rev.
389 34, 231–259. https://doi.org/10.1111/j.1574-6976.2009.00190.x
390 Thompson, F.L., Thompson, C.C., Swings, J., 2003. Vibrio tasmaniensis sp. nov., isolated from
391 Atlantic Salmon (Salmo salar L.). Syst. Appl. Microbiol. 26, 65–69.
392 https://doi.org/https://doi.org/10.1078/072320203322337326
393 Van der Henst, C., Scrignari, T., Maclachlan, C., Blokesch, M., 2016. An intracellular
394 replication niche for Vibrio cholerae in the amoeba Acanthamoeba castellanii. ISME J.
395 10, 897–910. https://doi.org/10.1038/ismej.2015.165
396 Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living
397 amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and
398 Sappinia diploidea. FEMS Immunol. Med. Microbiol. 50, 1–26.
399 https://doi.org/10.1111/j.1574-695X.2007.00232.x
400 Young, N.D., Cooper, G.A., Nowak, B.F., Koop, B.F., Morrison, R.N., 2008a. Coordinated
401 down-regulation of the antigen processing machinery in the gills of amoebic gill
402 disease-affected Atlantic salmon (Salmo salar L.). Mol. Immunol. 45, 2581–2597.
403 https://doi.org/10.1016/j.molimm.2007.12.023
404 Young, N.D., Dyková, I., Nowak, B.F., Morrison, R.N., 2008b. Development of a diagnostic
405 PCR to detect Neoparamoeba perurans, agent of amoebic gill disease. J. Fish Dis. 31,
406 285–295. https://doi.org/10.1111/j.1365-2761.2008.00903.x
407 Zhang, X., Li, K., Wu, S., Shuai, J., Fang, W., 2015. Peptide nucleic acid fluorescence in-situ
408 hybridization for identification of Vibrio spp. in aquatic products and environments. Int.
409 J. Food Microbiol. 206, 39—44. https://doi.org/10.1016/j.ijfoodmicro.2015.04.017
410

Figure legends

Figure 1. *Neoparamoeba perurans* trophozoite and bacteria from *in vitro* laboratory culture. The image demonstrates the bacteria present in *N. perurans* culture. The amoebae and bacteria were collected from a Malt-yeast Agar culture and transferred to a glass slide for imaging on an inverted microscope.

Figure 2. Proportional diversity of bacterial genera sequenced across various *P. perurans* culture conditions. The *Vibrio* genus occurred in the highest proportions in the sequences from day 0, day 1, day 1 PS (cultured with 125μg Pencillin-Streptomycin), and filtered amoebae (F1) samples. There was also a substantial proportion of *Vibrio* sequences in the day 7 and 1-month samples. The second largest proportion of sequences were attributed to the Rhodobacteriaceae in day 0, day 1, day 1 PS, and day 7 samples. *Vibrio* was also present in the 1-month (M1) sample, with a lower proportion of sequences within this sample. There was a small proportion of the *Halomonas* genus within the day 0, day 1, day 1 PS, and day 7 samples, while the 1-month sample had a larger proportion of sequences for this genus. The *Rhodospirillaceae* genus had the second largest proportion of sequences in the day 0, day 1, day 1 PS, and the largest proportion in the day 7 sample.

Figure 3. FISH Staining of *Vibrio* in *N. perurans* using cyanine 5 labelled PNA Probe. Cyanine 5 staining (in yellow) was observed within the *N. perurans* trophozoites. The staining was primarily within *N. perurans*, with limited extracellular bacterial cyanine 5 staining, indicating that *Vibrio* is contained within the amoeba. The DAPI staining (in light blue) shows the various extracellular bacteria present on the surface of the well.



437
438
439 **Figure 1**

440

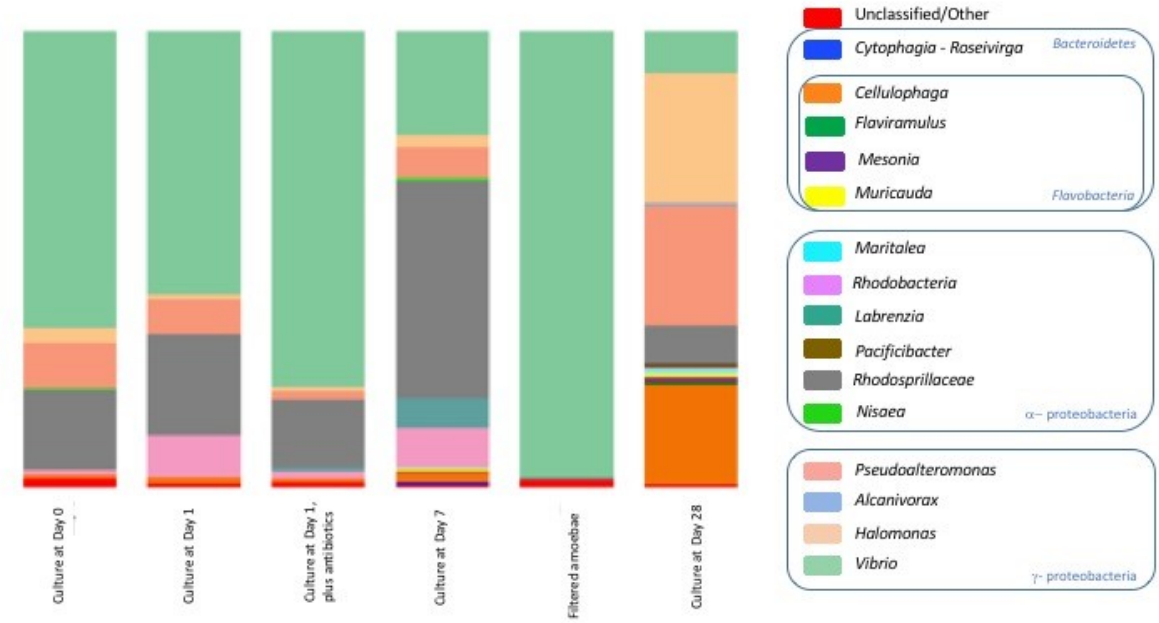

Culture at Day 0
Culture at Day 1
Culture at Day 1, plus antibiotics
Culture at Day 7
Filtered amoebae
Culture at Day 28
Unclassified/Other
Cytophagia - Roseivirga
Bacteroidetes
Cellulophaga
Flaviramulus
Mesonia
Muricauda
Flavobacteria
Maritalea
Rhodobacteria
Labrenzia
Pacificibacter
Rhodosprillaceae
Nisaea
α– proteobacteria
Pseudoalteromonas
Alcanivorax
Halomonas
Vibrio
γ- proteobacteria

Figure 2

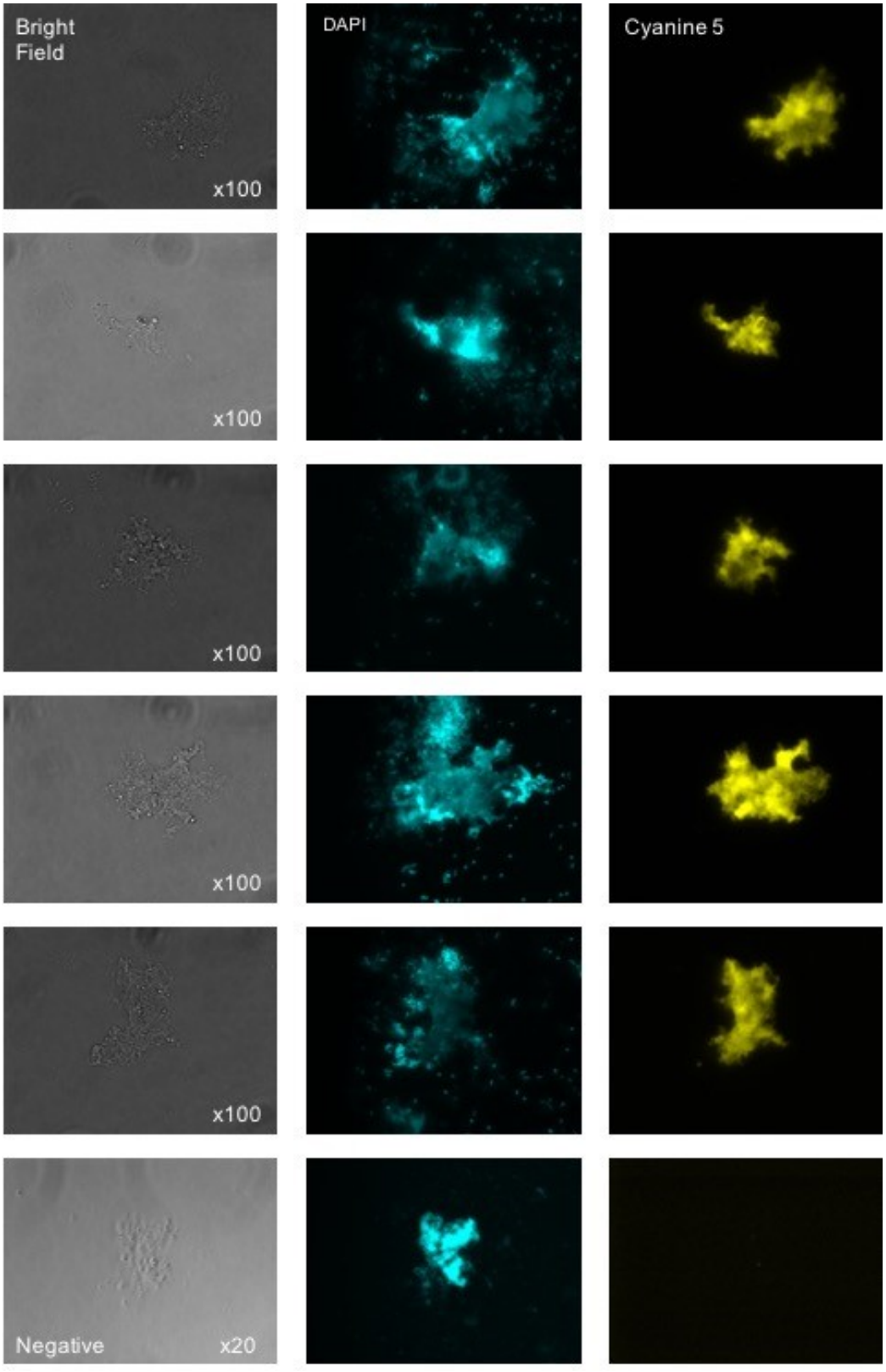

Bright Field
DAPI
Cyanine 5
x100
x100
x100
x100
x100
Negative
x20

Figure 3

450 Table 1
451
452

Genus	**Class**	**Habitat (reference)**
Pseudoalteromonas	γ- proteobacteria	Marine
Paraglaciecola or *Glaciecola* or *Alteromonas*	γ- proteobacteria	Antarctic sea/ marine
Halomonas	γ- proteobacteria	Hypersaline environment
Labrenzia or *Polymorphum*	α- proteobacteria	Hypersaline environment
Celeribacter or *Marivita*	α- proteobacteria	Marine
Cellulophaga	*Flavobacteria*	Marine
Thalassospira	*Flavobacteria*	Marine
Muricauda	*Flavobacteria*	Marine
Mesonia	*Flavobacteria*	Marine

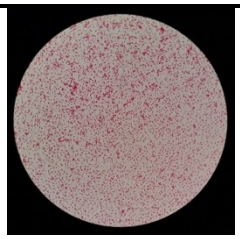
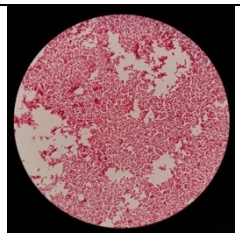
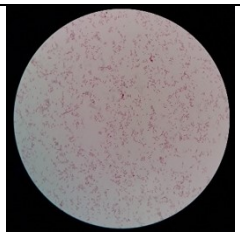
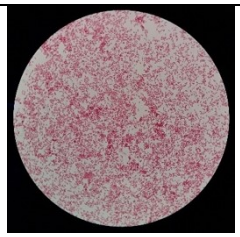
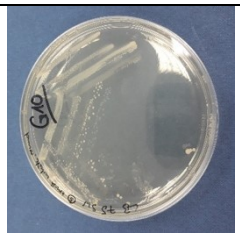
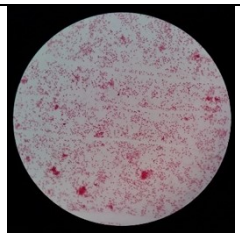
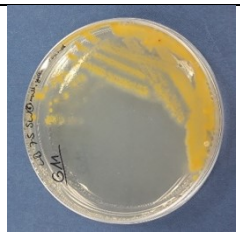
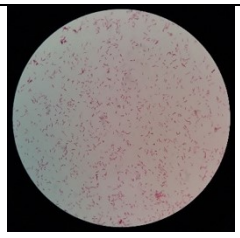
453 Table 2

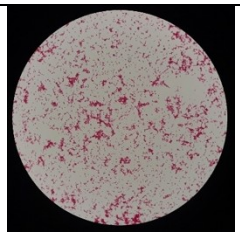
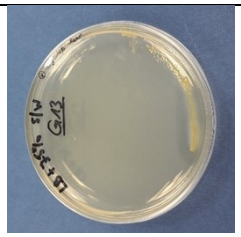
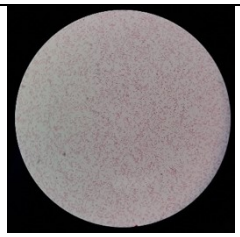
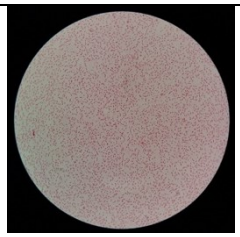
Culture time point and condition	*% of reads*
Day 0	60.3%
Day 1	53.6%
Day 1 (addition of Penicillin/Streptomycin)	71%
Day 7	21%
Day 1 - *Filtered amoebae*	90%
Day 28	8.9%

454
455

Supplementary material

Table A Individual isolation and sequencing of bacteria present in *P. perurans* culture

Genus	Morphology	Gram Stain
Pseudoalteromonas		
Paraglaciecola or *Glaciecola* or *Alteromonas*		
Halomonas		
Labrenzia or *Polymorphum*		
Celeribacter or *Marivita*		
Cellulophaga		

Thalassospira		
Muricauda		
Mesonia		

459
460

Table B

DNA Sample	Taxonomic Level	Match	Percentage Match (%)
Filtered Sample	g	*Vibrio*	90.6
	s	*Vibrio splendidus*	7.0
	s	*Vibrio crassostreae*	0.6
	s	*Vibrio* sp.	0.4
	f	*Vibrio tasmaniensis*	0.3
	s	Rhodospirillaceae	0.2
	s	*Vibrio lentus*	0.2
	s	*Vibrio* sp. B131a	0.2
	s	*Pseudoalteromonas* sp. HG03	0.1